A protocol for polymerase chain reaction detection of *Enterococcus faecalis* and *Enterococcus faecium* from the root canal

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Abstract

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Aim The present study was set up to develop a protocol for detection of *Enterococcus faecalis* and *Enterococcus faecium* from the root canal.

Methodology A collection of type strains and clinical isolates of *E. faecalis* and *E. faecium* was used. Specific polymerase chain reaction (PCR) primers targeted against the 16S/23S rDNA intergenic region were used and PCR reactions were set up. PCR products were run on TBE-agarose gel and analysed. The sensitivity of the PCR system was studied using serial dilutions of (i) bacterial DNA and (ii) bacterial cells from *E. faecalis*. The specificity of the identification was tested against closely related species.

Results All strains of *E. faecalis* and *E. faecium* produced identical amplicon profiles composed of two major bands corresponding to sizes of 320 and 420 bp. When amplifying DNA of higher purity, a third band of 600 bp became evident as well. Closely related species demonstrated single bands of various sizes and were easily distinguished from enterococci. The detection level of DNA from serial dilutions of DNA was 10^{-13} g. The DNA extraction protocol from bacterial cell suspensions resulted in a detection level of 10 bacterial cells per sample.

Conclusions The present study demonstrated a good potential for using PCR technology in the detection of *E. faecalis* and *E. faecium* from root canal samples. With a high specificity the methodology was able to detect 10 cells of *E. faecalis*.

Keywords: enterococci, microbiology, PCR, root canal therapy.

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Introduction

Endodontic treatment of teeth with apical periodontitis is directed toward eradication of the intracanal microorganisms. Hence, the efficacy of various combat regimes is often assessed by sampling the root canal for the presence of persisting microbes. Traditionally, identification of microorganisms in the samples has been carried out through various cultivation procedures. However, the accuracy of this methodology has been questioned and the risks of obtaining false positive and false negative recordings have been pointed out (Bender *et al.* 1964, Engström 1964, Möller 1966, Mikkelsen & Theilade 1969, Morse 1970, Zielke *et al.* 1976, Safavi *et al.* 1985, Reit & Dahlén 1988, Molander *et al.* 1990, Reit *et al.* 1999). Recently, there has been a focus on the influence of antibacterial dressings on the results of cultivation (Reit *et al.* 1999). For example, the chemical effects of a substance may cause a temporary loss of the multiplying capacity of surviving microorganisms, resulting in false negative observations. In addition, medicament remnants may enter a sample and inhibit microbial growth in the laboratory and result in a low diagnostic sensitivity. In order to increase the sensitivity of intracanal sampling, other methods of microbial detection and identification need to be explored.

In root canal microbiology alternative diagnostic methods have received limited attention. Nevertheless, when exploring various methods to identify

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periodontopathic bacteria Ashimoto *et al.* (1996) found polymerase chain reaction (PCR) to have a higher diagnostic accuracy than culture procedures. PCR has been described to amplify genomic sequences more than 10 million times (Mullis *et al.* 1986, Saiki *et al.* 1988) and to have a potential detection level of 10 bacterial cells (Zambon & Haraszthy 1995). Since the method is not dependent on bacterial growth, it may be suitable for analysis of the post-treatment intracanal microbiota.

Amongst bacteria resisting endodontic treatment procedures the frequency and role of enterococci have recently regained considerable attention (Gomes *et al.* 1996, Sirén *et al.* 1997, Molander *et al.* 1998, Sundqvist *et al.* 1998). PCR has been used extensively for speciation of enterococci, identification of virulence genes and for detecting the drug resistance of enterococci (Dutka-Malen *et al.* 1995, Tyrell *et al.* 1997, Shepard & Gilmore 1997, Hirakata *et al.* 1997, Monstein *et al.* 1998), but studies focusing on enterococci detection seem to be lacking. Therefore, the aim of the present study was to explore the potential use of PCR in diagnostic root canal microbiology by developing a protocol for the detection of *E. faecalis* and *E. faecium*.

Materials and methods

Bacterial strains

Type strains of *E. faecalis* (ATCC 19433, CCUG 19916) and *E. faecium* (ATCC19434, CCUG 542) were available from the Göteborg University Culture Collection (CCUG). In addition, four isolates of *E. faecalis* (OMGS 349/98, OMGS 350/98, OMGS 367/98, OMGS 1/97) recovered from infected root canals were also included (Dahlén *et al.* 2000). OMGS (Oral Microbiology, Göteborg, Sweden) strains are own isolates, if not CCUG, ATCC or NCTC is indicated. Prior to use these strains were transferred by means of sampling solution (VMGA I, Dahlén *et al.* 1993) from the lyophilized stage onto blood agar plates for incubation overnight in 37°C and air. DNA was prepared both directly from 'fresh' cultures and from strains kept frozen.

DNA preparation

(i) For the serial dilutions of chromosomal DNA from 10^7 cells, DNA was simply extracted by boiling for 5 min. (ii) To mimic a clinical sample, serial dilutions of *E. faecalis* cells in TE buffer (10 mmol L^{-1} Tris-HCl, 1 mmol L^{-1} EDTA, pH 8.0) were prepared, ranging from 10^7 to 10 per 100 μL; samples were processed in triplicates. DNA was extracted from these samples by using the Wizard Genomic DNA Purification System (Promega, Madison, WI, USA), except as noted according to the manufacturer's instructions, scaled down to a sample size of 100 μL. This kit uses a salt-based, selective precipitation step to remove proteins and cell debris. Phenol-chizam extraction was thus not required to obtain pure DNA. Initial cell wall degradation was performed by adding lysozyme, 450 μg, achromopeptidase, 150 μg, and mutanolysin, 15 μg (all from Sigma Chemical Co., St. Louis, MO, USA), to the samples. The samples were incubated at 37°C for 1 h, after which DNA isolation proceeded according to the manufacturer's instructions. RNAse treatment of lysed cells was postponed, allowing the bacterial RNA to act as a carrier for the precipitation of the chromosomal DNA. In addition, 0.5 μg sonicated salmon sperm DNA (Stratagene, La Jolla, CA, USA) was added to each sample to act as carrier when precipitating DNA. DNA from these preparations were resuspended in 20 μL of TE buffer overnight at 4°C. The resuspended DNA was treated with RNAse A, 5 μg, for 45 min at 37°C. The entire 20 μL of purified chromosomal DNA was added to the subsequent PCR reaction.

PCR conditions

The chromosomal DNA was amplified using the primers CAA GGC ATC CAC CGT and GAA GTC GTA ACA AGG targeted against the 16S/23S rDNA intergenic region (Barry *et al.* 1991, Jensen *et al.* 1993). PCR reactions were set up containing 0.1 μmol L^{-1} of each primer, 0.2 mmol L^{-1} dNTPs, 3 mmol L^{-1} Mg^{2+} and 1.5 units of TaqGold polymerase (Perkin-Elmer, Foster City, CA, USA) in a volume of 50 μL and amplified using the following sequence: 95°C for 2 min succeeded by 40 cycles of 95°C 60 s, 55°C 60 s, 72°C 60 s followed by a final elongation step at 72°C for 10 min. As a positive control of the PCR reaction a type strain of *E. faecalis* (ATCC 19433, CCUG 19916) was used. A negative control devoid of template DNA was included in all experiments. All components used in preparation of DNA was also amplified in the same manner to ascertain that no contamination or cross reactivity had been introduced by the preparation method.

Electrophoresis and imaging

Polymerase chain reaction products were run on 1% or 2.5% TBE-agarose (Seakem GTG agarose, FMC Bioproducts, Rockland, ME, USA) gel and visualized by ethidium bromide staining under UV light and photographed.

Subcloning and sequencing

When PCR amplifying highly purified chromosomal DNA from *E. faecalis*, a previously undetected band of 600 bp became evident. To ascertain the origin of this band it was excised from the gels and the DNA purified using the QIAEX II gel extraction kit (Qiagen, Valecia, CA, USA). Purified PCR product was cloned into the pGEMT vector (Promega, Madison, WI, USA) and transformed into JM109 competent cells (Promega) according to the manufacturer's instructions. Positive colonies were isolated and plasmids purified with the Wizard Plus Sv Minipreps (Promega) plasmid purification system. Clones were sequenced by cycle sequencing using the Big Dye terminator sequencing kit (ABI Prism, Perkin Elmer, MA, USA) and T7 and Sp6 sequencing primers (Promega). Reactions were then analysed on an ABI 377 automated DNA sequencer (Perkin Elmer). Four individual clones were sequenced.

Accuracy of the PCR system

Using strains of *E. faecalis*, the sensitivity of the PCR system was studied by (i) titrating bacterial suspensions of 10^7 cells mL^{-1}, estimated by turbidimetry at 605 mm, in 10-fold dilutions series, and by (ii) 10-fold dilution series of extracted DNA from 10^7 cells. The original suspension and the dilutions were thoroughly mixed by vortexing. The series were run in triplicates.

The specificity of the method was tested against type strains of *Streptococcus equinus* (OMGS 2297), *Streptococcus uberis* (OMGS 2999), *Streptococcus milleri* (OMGS 1773), *Streptococcus anginosus* (OMGS 2479, NCTC 10713), *Streptococcus pyogenes* (OMGS 1775, CCUG 23117), *Streptococcus mutans* (OMGS 2428, ATCC 25175), *Streptococcus salivarius* (OMGS 2293), *Streptococcus sanguis* (OMGS 2478, ATCC 10556), and *Gemella morbillorum* (OMGS 2415).

Results

All strains of *E. faecium* and *E. faecalis* produced identical amplicon profiles with two major bands in positions corresponding to 320 and 420 base pairs (bp) (Fig. 1). When amplifying DNA of higher purity prepared from serial dilutions of *E. faecalis* using the Wizard Genomic DNA Purification System (Promega), a third band of 600 bp became evident (Fig. 2). The sequence of this previously undetected 600 bp amplicon was determined and aligned against the Genbank database. It matched no previously identified sequence but showed a partial

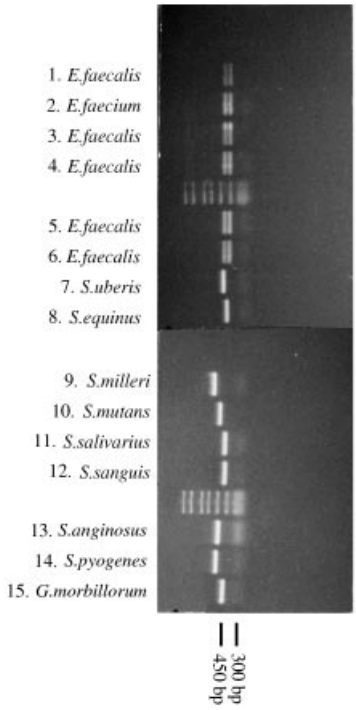

Figure 1 PDR profiles of investigated strains. Lanes 1, *E. faecalis* (ATCC 19433, CCUG 19916); 2, *E. faecium* (ATCC 19434, CCUG 542); 3, *E. faecalis* (OMGS 350/98); 4, *E. faecalis* (OMGS 266/98); 5, *E. faecalis* (OMGS 349/98); 6, *E. faecalis* (OMGS 36798); 7, *S. uberis*; 8, *S. equinus*; 9, *S. milleri*; 10, *S. mutans*; 11, *S. salivarius*; 12, *S. sanguis*; 13, *S. anginosus*; 14, *S. pyogenes*; 15, *G. morbillorum*.

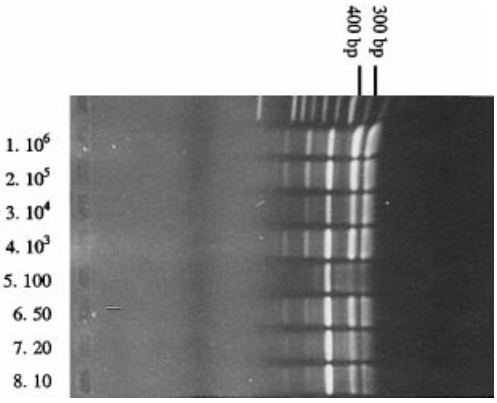

Figure 2 PCR profiles of 10-fold serial dilution series of cells of *E. faecalis* (ATCC 19433, CCUG 19916).

(nucleotides 180–360 of 600) 90% homology to *Xanthomonas campestris* 16S-23S intergenic spacer DNA (Genbank acc. No. AF279434.1). *S. equinus*, *S. uberis*, *S. milleri*, *S. anginosus*, *S. pyogenes*, *S. mutans*, *S. salivarius*,

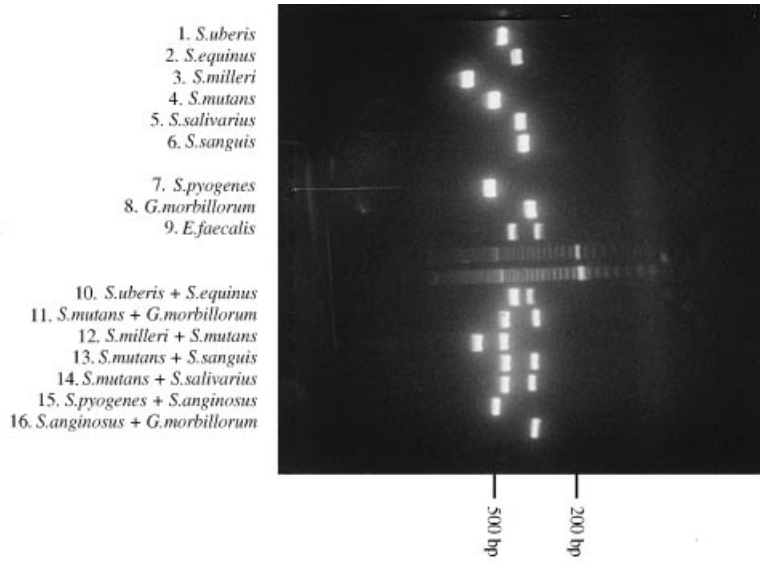

Figure 3 PCR profiles of investigated strains and mixtures of strains run on a 2.5% TBE-agarose gel. A 20 bp interval ladder, ranging from 20 to 1000 bp, was used.

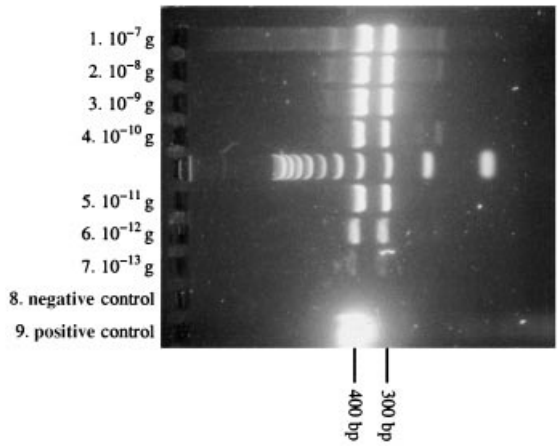

Figure 4 PCR profiles of 10-fold serial dilution series of extracted DNA from 10^7 cells of *E. faecalis* (ATCC 19433, CCUG 19916).

S. sanguis and *G. morbillorum* were associated with single bands in various positions (Figs 1, 3).

The DNA prepared directly from pure cultures of *E. faecalis* produced identical amplicon profiles as DNA prepared from frozen isolates.

The detection level of DNA in serial dilutions was 10^{-13} grams (Fig. 4). The protocol for extraction of DNA resulted in a detection level of 10 cells (Fig. 2).

Discussion

Polymerase chain reaction amplification of the 16S/23S ribosomal spacer region (ITS-PCR) produced characteristic and identical amplicon profiles for *E. faecalis* and *E. faecium*. When the same pair of primers were used on DNA extracted from *S. equinus*, *S. uberis*, *S. milleri*, *S. mutans*, *S. salivarius*, *S. sanguis*, *S. anginosus*, *S. pyogenes* and *G. morbillorum* profiles were produced that were easily distinguished from the enterococci when run in separate lanes to high separation on 2.5% TAE agarose gels. Enterococcus species have, until recently, been classified as streptococci, according to Lancefield as group D. *S. uberis* and *S. equinus* are still classified as streptococcal species, belonging to Lancefield group D (Hardie 1986). Thus *S. equinus* and *S. uberis* have a close relationship to enterococci and if these two species should be PCR amplified together and subsequently run in the same lane of the gel they could be expected to resemble the two-band pattern of *E. faecalis* and *E. faecium*. However, optimal electrophoresis conditions clearly separated these two species from the enterococci. Moreover, *S. equinus* and *S. uberis* are not relevant in root canal infections but were included in order to challenge the methodology. It thus seems as if the pair of primers used in this study is suitable for identification of enterococci at the genus level. This is in concordance with the findings of Tyrell *et al.* (1997).

When PCR amplifying highly purified *E. faecalis* DNA a third amplicon of 600 bp became evident. The sequence of this DNA fragment showed a partial strong homology to a 16S-23S intergenic spacer sequence from the proteobacter *X. campestris*. This high homology to another 16S-23S intergenic spacer sequence leads us to conclude that this is probably a third *E. faecalis* 16S-23S intergenic

spacer sequence even though the *E. faecalis* and *X. campestris* are only distantly related. To unequivocally assign this DNA sequence as an *E. faecalis* 16S-23S intergenic spacer, identification of flanking regions and Southern blots on *E. faecalis* need to be performed. Meanwhile, in the scope of the present study, the 600 bp amplicon poses no problem to identification and detection of *E. faecalis* by the PCR technique developed.

In a root canal sample of the posttreatment microbiota a low number of microorganisms can be expected. Consequently, a very low detection level of the identification methodology is essential. Crucial for DNA-techniques is the extraction of DNA from the cells. Lysis of the cells by boiling, a technique favoured in identification of periodontopathic bacteria (Ashimoto *et al.* 1996, Papapanou *et al.* 1997), was not successful in our study. In contrast to samples obtained from the negotiated root canal, samples from gingival pockets contain a large number of microorganisms. Also, in that context the species of interest are anaerobic and mostly Gram negative. Such bacteria are easily disrupted by physical influence and sufficient amounts of DNA are rather easily extracted. In the present study a great number of protocols for extraction of DNA from serial dilutions of cells of *E. faecalis* were unsuccessfully tested. Traditional techniques such as boiling, enzymatic cell lysis followed by proteinase K digestion and phenol-chizam extraction gave detection levels in the range of hundreds to thousands of bacteria per sample. Finally, using the method described above, a detection level of 10 cells was reached. This level is in concordance with what has been described elsewhere (Zambon & Haraszthy 1995). The practical results correspond to the theoretically calculated potential of the PCR protocols to detect approximately 20 bacterial genomes from 10^{-13} grams of DNA. Using conventional culturing identification methodology on plaque samples, Loesche *et al.* (1992) reported a detection level of $\geq 2 \times 10^3$ cells. Zambon & Haraszthy (1995) detected 10^4–10^5 cells using non-selective media and 10^3 cells when selective media were used. Contrasting these findings from mixed samples, Möller (1966), using broth, was able to disclose $\leq 5 \times 10^1$ cells for several root canal species when cultured as monocultures. Although enterococci easily grow on selective media, PCR might be the slightly superior technology regarding the detection level. However, the advantages of PCR over culturing above all are associated with its low sensitivity to physical and chemical influence. In a clinical situation the use of various medicaments like chloroform, interappointment dressings and irrigants are unlikely to bias the test performance.

An apparent limitation of a species specific PCR-based bacterial detection is its inability to detect 'unexpected' bacteria. In other words, the technique can only identify selected microorganisms for which specific primers are available. Moreover, it may not be as useful for 'broad-range' microbiological analysis of the root canal, although a few different species can be simultaneously detected from samples of small volume by utilizing a multiplex PCR protocol. Such broad range detection is possible using primer pairs targeted to conserved gene sequences. Extensive subcloning and sequencing must then, however, be performed to identify species present in the sample, which, from practical reasons, will limit its use in a clinical situation. In addition, PCR does not discriminate dead from viable cells. The dead microbe will degrade in the canal due to lyzosomal activities but the fate and significance of DNA in a non-vascular confinement is poorly explored.

Conclusions

In conclusion, the present study demonstrated a potential use of PCR technology for the detection of *E. faecalis* and *E. faecium* in root canal samples. Clinical studies directly comparing PCR and culturing of samples are now indicated.

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